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Characterization and Identification of Bacterial Flora from Infected Equine Hooves

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Characterization and Identification of Bacterial Flora from Infected Equine Hooves

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By

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Abstract

The aim of this research was to characterize and identify the bacterial flora associated with a specific hoof infection in a population of horses in the Rochester NY area. Samples from horses showing symptoms of infection were first grown on Brain-Heart Infusion (BHI) solid medium, a rich medium that allow growth of all bacteria. Forty different bacterial colonies were obtained and characterized microscopically and biochemically. We have identified normal inhabitants of the skin such as *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Micrococcus varians,* and *Micrococcus luteus*. Bacteria commonly found in soil such as *Bacillus megaterium, Bacillus azotoformans, Bacillus insolitus*, and *Bacillus popilliae* were also identified along with *Enterococcus faceium*, which is found in the gastrointestinal tract of mammals (1, 2, 3). Interestingly bacteria such as *Corynebacterium xerosis*, known to cause animal diseases were also isolated from infected horses (4). Several of these bacteria were confirmed by sequencing 16S ribosomal DNA. Surprisingly *Enterococcus gallinarum*, *Bacillus subtilis*, and *Macrococcus* were also identified. *Bacillus subtilis* is commonly found in soil; therefore its presence is understandable. *Enterococcus gallinarum* is very rare and is found in the intestinal tract of mammals. One species of *Macrococcus, Macrococcus epuipercicus,* has been found on horse's skin (5). The bacteria responsible for the hoof infection have yet to be identified, although it is hypothesized that the bacteria are anaerobic and prefer moist environments.

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Acknowledgements

I want to thank my thesis advisor Dr. Pelletier for helping to develop my thesis and guiding my work in the laboratory. Without his help I would not have been able to complete my research. I also want to thank my Farrier, Greg Zoller, for proposing the idea to identify the infection causing bacteria in my horses' hooves and aiding me in the collection of my samples. Lastly, I want to thank my parents for supporting my interest in science and my love of horses.

Introduction

Hoof infections in horses are a very common problem. The majority of infections are mild and can be treated without consequences, although some cases result in chronic lameness and can be life threatening (6). The hoof is argumentatively the most important component of a horse's anatomy. It protects the internal structures of the foot such as the coffin bone (7). The major components of the hoof are the coronary band, wall, sole, white line, and frog (7).

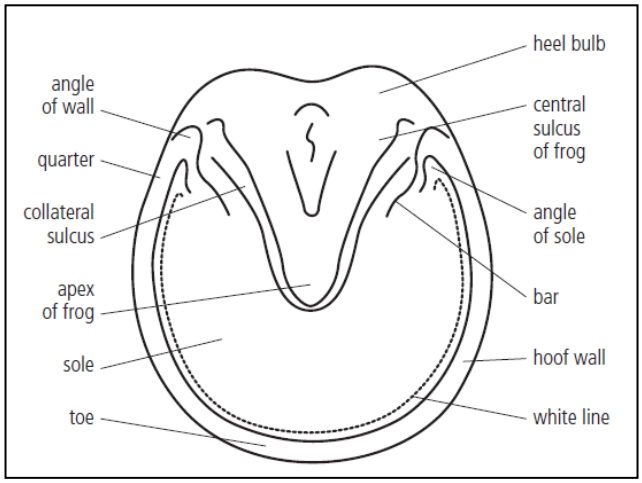

Figure 1: Ground view of Hoof (7)

The coronary band is the junction point of the hoof with the skin and is responsible for the growth of the hoof wall (7). The wall is the hard exterior of the hoof and connects with the underside of the hoof, the sole, at the white line (7). Projecting into the sole from the heel of the hoof is a wedged shaped structure called the frog (7). The wall and the frog are the weight-bearing surfaces of the hoof which is the result of the concaveness of the sole (7).

The hoof substance is made up of tubular and intertubular horn (7). The proportions of tubular to intertubular vary with respect to location (7). For instance, the wall of the hoof is predominantly tubular, but the frog is predominantly intertubular (7). The tubular horn is produced by the hair-like dermal

papillae of the coronary band, sole, and frog (7). The intertubular horn is the product of the hollows that form between the dermal papillae (7). Damage to the coronary band causes defective growth of the hoof (7). Damage can be a result of physical trauma along with abscesses.

An abscess is basically a pocket full of pus within the hoof (7). It is caused by bacteria entering through a wound in the sole and traveling up the white line (7). Abscesses can also be caused by deep bruises to the sole of the hoof (7). In either case, bacteria become trapped inside the hoof and pressure builds causing pain (7). The trademark symptom of an abscess is severe lameness (7). In some cases, a horse may actually walk three legged. This unequal weight distributions can cause laminitis which left untreated can lead to founder (7). Founder is especially damaging to the overall health of the horse because it causes rotation of the coffin bone within the hoof and leads to prolonged lameness (7). In severe cases, the horse is euthanized. Abscesses are treated by opening up the pocket of pus (7). This relieves the built up pressure and allows drainage of the bacteria (7). Additionally, the hoof can be soaked in Epsom salts and wrapped to aid in healing. Abscesses can also damage the coronary band when the bacteria tracks up the white line and breaks out at the coronary band (7).

Figure 2: Abscess that has erupted from coronary band (7)

Another common hoof infection is thrush. Thrush is an infection of the central and lateral sulci (clefts) of the frog of the horse's foot (8). The name thrush implies a fungal infection, although, thrush in a horse's frog most often involves bacteria (8). One species of bacterium, *Fusobacterium necrophorum*, is particularly aggressive, invading and destroying the frog, sometimes exposing the deeper sensitive tissues (8). A combination of factors makes domestic horses susceptible to thrush such as soft or damp footing, improper trimming, and a diet rich in sugars and carbohydrates (7). Thrush is treated by removing overgrown areas of the frog and sole as well as scrubbing the infected area with povidine iodine or hydrogen peroxide (7).

Lastly, white line disease is the separation of the hoof wall from the sole which allows bacteria and/or fungi to invade the resulting space (7). Horses are more susceptible to white line disease in moist or drought conditions (7). Severe white line disease can lead to chronic laminitis in horses (7). Treatment for white line disease involves removing the infected areas of the white line and applying an anti-bacterial such as iodine (7).

Figure 3: Hoof infection with white line disease (7)

The horses involved in this research both have a history of abscesses, thrush, and white line disease. The aim of this research was to identity the bacteria causing the hoof infection that has weakened

the white line, produced a foul smelling odor and black residue, and led to cracks and abscesses in the horses' hooves.

Methods and Materials

Sample collection:

Samples were collected from the infected hooves of two horses on September 8th, 2012. The first horse was a 20 year old Paint mare and the second was a 14 year old Thoroughbred gelding. Both horse reside at the same location and are therefore exposed to the same elements. The samples were taken with the assistance of Greg Zoller, Farrier. The infected areas were cleaned out using a hoof knife and sterile swabs were used to sample the area. The swabs were placed in sterile tubes containing saline solution and refrigerated until plating. A total of 11 samples were taken which included 9 samples from infected regions on the hoof, 1 control, and 1 sample from a hoof clipping to establish the general flora of the hooves.

Preparation of Plates:

Brain and Heart Agar:

-18.5g Brain/Heart Infusion

-7.5g Agar

-500ml Water

The Brain/Heart Infusion and Agar were dissolved in water and then autoclaved. The Brain and Heart Agar was distributed into 20 plates.

Isolation Media for *Fusobacterium necrophorum* (9):

-16g Trypticase

-2.6 g PamR. digesd casein

-1.4g Yeast extract

2g Thiotone

-0.5g Glucose

-40µl $MgSO_4$ (12%)

-2.5g Na_2HPO_4

-8.3g Agar

-415ml Water

The ingredients were combined, autoclaved, and allowed to cool overnight (9). The media was warmed up in a water bath and 1.35ml of phenethyl alcohol, an egg yolk, and 45 ml of 9% saline solution were added (9). Lastly, 0.2g Crystal violet was dissolved in 25ml sterile distilled water and added to the medium (9). The medium was equally distributed in 30 plates.

Plating Samples:

The 11 samples were plated on Brain and Heart Agar and Isolation Media for *Fusobacterium necrophorum*. There were two sets of Brain and Heart Agar plated; one for aerobic conditions and the other for anaerobic. The aerobic Brain and Heart Agar and Isolation Media were incubated at 37˚C for 48 hours. The anaerobic Brain and Heart Agar plates were placed in an anaerobic chamber and incubated at 37˚C for 48 hours.

Isolating Bacteria:

After the two day incubation period, each plate was investigated and observations recorded. The bacteria on each plate were replated using the three way streak method and incubated at 37˚C overnight. This included the bacteria on the anaerobic plates as well.

Identifying Bacteria Method 1:

A gram stain was performed on each bacterium and the results recorded. These results were used to determine which pathway of biochemical tests should be taken in order to identify the bacterium. These tests included catalase production, mannitol fermentation, glucose fermentation, novobiocin sensitivity, starch hydrolysis, citrate, gelatinase, Voges–Proskauer, swollen cell, hemolysis, and spore formation. The results of these tests led to tentative identities for the bacteria.

Catalase

To test for the presence of catalase a small amount of bacteria was transferred onto a glass slide. A drop of 3% hydrogen peroxide was added to the bacteria and if the bubbles were produced then the bacteria tested positive.

Mannitol Fermentation

The test for mannitol fermentation involved plating the bacteria sample onto a Mannitol Salt Agar, MSA, plate and incubating the plate over night at 37˚C. MSA contains phenol red, an indicator that turns yellow when acid is produced and the pH of the medium gets below 6.8. If the plate changed color from pink to yellow then the bacteria was able to ferment mannitol.

Glucose Fermentation

The test for glucose fermentation was similar to the test for mannitol fermentation. A small amount of the bacteria was added to a tube containing glucose and phenol red and incubated overnight at 37˚C. If the contents of the tube changed from purple to yellow then the bacteria was able to ferment glucose.

Novobiocin Sensitivity

To test for novobiocin sensitivity, bacteria were plated over an entire Brain and Heart plate and a disk containing 30µg of novobiocin was placed in the middle. The plate was incubated overnight at 37˚C

and was inspected the next day for lack of growth around the novobiocin disk. If the lack of growth exceeded 22mm diameter then the bacteria were inhibited by the novobiocin and therefore were sensitive to the antibiotic.

Starch Hydrolysis

The ability of a bacterium to hydrolyze starch was tested by plating a streak of the bacteria onto a starch plate and incubating overnight at 37˚C. The following day iodine was poured onto the plate and then drained. If the bacterium was able to hydrolyze the starch then a halo would appear around the bacterial growth.

Citrate

The citrate test was used to determine the ability of a bacterium to use citrate as it sole source of carbon and energy. The bacteria were plated in a slanted citrate tube, using the stab and streak method, and incubated overnight at 37˚C. If the bacteria grew then the bacteria tested positive and if there was a color change from green to blue it meant that the bacteria also produced acid.

Gelatinase

The test for gelatinase was performed by inoculating a tube of gelatin, using stab method, with the desired bacterium and incubating the tube overnight at 37˚C. The next day the tube was placed on ice and left for 30 minutes. If the contents of the tube remained liquid then the bacteria tested positive for gelatinase, although if the contents of the tube became solid then the tube was incubated another night at 37˚C and the test repeated. The tube was re-incubated because the enzyme gelatinase can take awhile to digest gelatin. If the tube remained solid on the next day then the bacteria tested negative for gelatinase.

Voges–Proskauer

The Voges-Proskauer test was used to determine the presence of acetoin in a bacterial culture. The test was performed by inoculating a tube of Voges-Proskauer broth and incubating overnight at 37˚C. Following incubation alpha-naphthol and potassium hydroxide were added to the tube. A color change to pink indicated a positive result.

Swollen Cell

To determine the presence of a swollen cell the bacterium was placed on a glass slide, stained, and investigated under a microscope. Swollen cells appeared rounder and larger than the normal cells.

Hemolysis

The test of hemolysis determines a bacterium's ability to breakdown red blood cells. The bacterium was plated onto a Blood agar plate and incubated overnight at 37˚C. The following day the growth on the plate was investigated. There were three possible results of hemolysis; alpha, beta, and gamma. Alpha hemolysis or partial hemolysis was characterized by the agar surrounding the bacterial growth appearing dark and greenish. Beta hemolysis or complete hemolysis resulted in a clear halo around the bacterial growth. Gamma hemolysis was when the bacterium was unable to digest red blood cells. In this instance, the agar remained unchanged.

Spore Formation

To test for spore formation the bacteria was grown in a tube containing nutrient broth for one day at 37˚C. The tube was then heated in a 70˚C water bath for 10 minutes. The broth was plated onto a Brain and Heart agar and incubated overnight at 37˚C. If growth was seen on the plate the next day then the bacterium was able to form spores.

Identifying Bacteria Method 2:

In order to confirm the tentative identities of the bacteria the DNA from each bacterium was sequenced. DNA sequencing can be divided into several steps; isolating genomic DNA, amplification of DNA, gel electrophoresis, PCR cleanup, ligation, cloning DNA into plasmids, purifying plasmid DNA, and DNA sequencing.

The process of isolating the Genomic DNA started off by growing each bacterium in 3mL of Brain and Heart Broth (18.5g Brain and Heart Infusion and 500mL Water). The bacteria cells were then spun down at 13,000 x g for two minutes in a 1.5ml Eppendorf tube. The supernatant was removed and the pellet was suspended in 150µl of Wizard SV Lysis Solution. The contents were transferred to the wizard mini-column and collection tube apparatus. The apparatus was spun at 13,00xg for three minutes. The column was then removed to discard liquid and replaced into the collection tube. Next, 650µl of column wash solution was added to the column and spun at 13,00xg for one minute. The liquid in the collection tube was discarded. The wash process was repeated two more times. After the third wash, the collection tube was emptied and spun dry at 13,000 x g for two minutes. The column was removed from the collection tube and placed into a 1.5mL Eppendorf tube. Added to this was 250µl of Nuclease-Free water and 2µl of RNase A solution. This was incubated for two minutes at room temperature and spun at 13,000 x g for two minutes. The mini-column was discarded and the Genomic DNA collected in the Eppendorf tube was stored at -20˚C.

The master mix used for the amplification of the DNA consisted of:

-35µl 10x Buffer

-10.5µl 20mM dNTPs

-1.1µl FD1 (100µM)

-1.1µl rD1 (100µM)

-263.3µl Water

-4µl Phosphate DNA polymerase

The master mix, 90µl, was dispensed into PCR tubes along with 10µl of Genomic DNA. Then the PCR reaction was run.

1. 95˚C for 5 minutes
2. 95˚C for 2 minutes, 42˚C for 30 seconds, 72˚C for 4 minutes---35 cycles
3. 72˚C for 20 minutes
4. 10˚C for ∞

Gel electrophoresis was used to confirm the amplification of the DNA. The first well contained 5µl of 1KB ladder and the remaining wells contained 10µl of amplified DNA with 2µl of green buffer. The gel was covered with 600mL of 1x Running Buffer and run at 400 MA/125 Volts for 30 minutes.

Once the presence of the amplified DNA was confirmed, it was cleaned-up to remove any by-products of the PCR process. The amplified DNA was placed into 1mL eppendorf tubes along with an equal amount of binding solution. Then 5µl of silica powder was added to each tube and incubated at 55˚C for five minutes with vortexing occurring at two minutes. The tubes were spun at 13,000 x g for five seconds and the supernatant discarded. The pellet left in each tube was vortexed with 500µl of ice-cold wash buffer and spun at 13,000 x g for five seconds. The supernatant was discarded and the wash process repeated two more times. After the third wash the pellet in each tube was spun dry at 13,000 x g for five seconds. The DNA was eluted by resuspending the pellets in 15µl of TE Buffer, incubating for five minutes at 55˚C, spinning at 13,000 x g for five seconds, and collecting the supernatant into a clean Eppendorf tube. The elution process was repeated and the supernatants combined. The DNA was stored at -20˚C.

The cleaned PCR was ligated in preparation for bacterial transformation. The ligation reaction, set up on ice, consisted of 10µl of 2X reaction buffer, 1µl of non-purified PCR product, 1µl of pJET1.2/blunt cloning vector (50ng/µl), 7µl of nuclease-free water, and 1µl of T4 DNA ligase. The ligation mixture was vortexed briefly, centrifuged at 13,000 x g for 5 seconds, and incubated at room temperature (22˚C) for five minutes. The ligation mixture was stored at -20˚C until bacterial transformation.

For bacterial transformation 50µl of *E. coli* DH5α cells were thawed on ice and transferred into a 14ml snap tube, also on ice, along with 5µl of the ligation mixture. The transformation mixture was vortexed briefly and incubated on ice for 30 minutes. After incubation, the transformation mixture was placed into a water bath at 42˚C for 45 seconds without shaking and replaced onto the ice for two minutes. Then, 500µl of room temperature SOC was added to the transformation mixture and incubated at 37˚C for 1 hour at 225rpm. Lastly, 100µl of the transformation mixture was plated on room-temperature LB-ampicilin plates and incubated overnight at 37˚C.

The plates were investigated the next day for the presence of isolated bacterial colonies. If such colonies grew then the colony was removed from the plate and placed into a tube containing 3ml of nutrient broth and 3µl of ampicilin. The tube was incubated at 37˚C overnight at 225rpm. The next day, the contents of the tube were centrifuged for two minutes at 13,000 x g in a 1.5ml Eppendorf tube. The supernatant was then discarded. The cells were resuspended in 250µl of Resuspension solution by vortexing. Once the cells were resuspended, 250µl of Lysis solution was added and vortexed. Next, 350µl of neutralization solution was added and the tube inverted 4 to 6 times. The tube was then centrifuged for five minutes at 13,000 x g. The supernatant was loaded into the Gene Jet Spin column and centrifuged for one minute at 13,000 x g. The column was washed twice by adding 500µl of wash solution to the column, centrifuging for one minute at 13,000 x g, and discarding the flow through. The column was spun dry for one minute in the centrifuge at 13,00xg. Lastly, to elute the purified DNA, the column was transferred to

a new 1.5ml Eppendorf tube along with 50µl of Elution buffer and was incubated for two minutes at room temperature. This was then centrifuged for two minutes at 13,000 x g and the flow through collected. The purified DNA was then stored at -20˚ until it was ready to be sent for DNA sequencing.

Before sending out the purified plasmid DNA to be sequenced, the oligonucleotide pJET-F: CGACTCACTATAGGGAGAGCGGC was added to the DNA. The samples were then sent out to be sequenced and the results came back in three to four days.

Results

The samples collected from the two horses produced fifty bacteria which were isolated and characterized. The fifty bacteria were isolated from growth on the Brain and Heart Agar plates. No bacteria grew on the Isolation Media for *Fusobacterium necrophorum*, therefore the bacterial infection was not thrush (8). Also it should be noted that these fifty bacteria included both the aerobic and anaerobic bacteria, although they are not separated because the anaerobic bacteria survived when grown in aerobic conditions. The fifty bacteria were characterized using macro and micro observations. The macro and micro observations showed that certain bacteria appeared more than once. The repeats were discarded. This left forty bacteria which went onto biochemical testing. The table below contains the results of the macro and micro observations for the forty bacteria.

Sample #	Colony Appearance	Gram Stain	Morphology/Arrangement
1	Creamy white, small	(+)	Cocci
2	Milky White	(+)	Medium Cocci
3	Milky white, small	(+)	Cocci, Chains and Clusters
4	Dry White	(+)	Medium Rods, Clusters
5	Dull, Yellow, Small	(+)	Cocci, Clusters
6	White, Small	(+)	Cocci, Clusters
7	Creamy White	(+)	Cocci, Clusters
8	Creamy White	(+)	Cocci, Clusters
9	Dull Yellow	(+)	Cocci Clusters
10	Milky Yellow, Small	(+)	Short Rods, Clusters
11	Creamy White	(+)	Short Rods, Clusters
12	Fibrous White	(+)	Large Rods. Chains
13	Milky Yellow, Small	(+)	Short Rods, Clusters
14	Peach, Small	(+)	Short Rods, Clusters
15	Dark Yellow	(+)	Medium Rods
16	Creamy White	(+)	Medium-Small Rods
17	Creamy White	(+)	Medium-Small Rods, Clusters
18	Milky Yellow, Small	(+)	Medium-Small Rods, Clusters
19	Yellow-White Streak	(+)	Rods, Clusters
20	Cream	(+)	Medium Rods, Clusters and Singular
21	Dry, Creamy Yellow	(+)	Medium-Small Rods, Clusters
22	Milky White	(+)	Short Rods
23	Creamy White, Radius=0.25cm	(+)	
24	Creamy White, Medium-Small	(+)	Short Rods, Clusters
25	Flat, Dull, White, Large	(+)	Rods, Clusters
26	Yellow	(+)	Short Rods
27	Creamy White, Small	(+)	Rods, Clusters
28	Peach, Small	(+)	Short Rods, Clusters
29	Milky White	(+)	Short Rods
30	Creamy White, Small	(+)	Very Short Rods, Clusters and Singular
31	Peach, Small	(+)	Very Short Rods
32	Creamy Light Peach	(+)	Very Short Rods
33	Yellow	(+)	Rods, Clusters
34	Creamy White	(+)	Rods
35	Dull Yellow	(+)	Cocci or Short Rods
36	Yellow	(+)	Short Rods, Clusters

37	Yellow	(+)	Cocci or Short Rods
38	Yellow	(+)	Cocci or Short Rods
39	Peachy Yellow, Small	(+)	Cocci or Short Rods, Clusters
40	Yellow, Small	(+)	Medium Shinny Rods

Figure 4: Table of macro and micro observations of forty bacterial colonies

In some cases, it was difficult to determine the morphology of the bacterial sample because the sample could be classified as cocci or small rods depending on the opinion of the observer. This uncertainty made biochemical testing more challenging. Each bacterial colony underwent a series of biochemical tests based on their morphology and arrangement. The results of the biochemical tests are listed below in the table.

Sample #	Catalase	Mannitol Ferm.	Glucose Ferm.	Novobiocin Sen.	Starch	Citrate	Gelatinase	VP	Swollen Cell	Hemolysis	Spore
1	+	-	+								
2	+	-		+							
3	+	-		-							
4					+	+		-	-		
5	+	-	-								
6	-	+ white	weak +							Gamma	
7	-									Gamma	
8	-									Gamma	
9	+	-		-							
10	+				-						
11	+				-						
12	-				-	-	-				
13	+				-						
14	+				-						
15	+				-						-
16	+				-						-
17	+				-						-
18	+				-						-
19	+				-						-
20	+				-						-
21	-										-
22	-					+					+
23	+				-						-
24	+				-						
25	+								-		+
26	+				-						-
27	+		-						-		+
28	+				-						-
29	+				-				-		+
30	+				-						-
31	+				-						-
32	+				-						-
33											
34	+				-						-
35	+	-	+								
36											
37											
38											
39											
40											

Figure 5: Table of the results of the biochemical tests

The results of the biochemical tests allowed us to determine the identity of most of the samples. Several of the bacterial samples did not undergo biochemical testing due to a lack of time. The table below lists the identity of the bacteria determined from the biochemical tests in the Crude ID column. The results from the biochemical tests showed that once again certain bacteria appeared more than once. This reduces the number of bacteria isolated to twenty. The second column of the table list the actual identity of the bacteria determined from sequencing the DNA. The sequencing of DNA was only completed for seven bacterial samples due to a lack of time and difficultly with the bacterial transformation.

Sample #	Crude ID	DNA Sequencing
1	M. varians	
2	S. epidermidis	
3	S. saprophyticus	Macrococcus
4	B. megaterium	
5	M. luteus	B. subtilis
6	E. faecium	E. gallinarum
7		E. gallinarum
8		E. gallinarum
9	S. saprophyticus	
10	C. xerosis	E. gallinarum
11	C. xerosis	
12	B. popilliae	
13	C. xerosis	C. xerosis
14	C. xerosis	
15	C. xerosis	
16	C. xerosis	
17	C. xerosis	
18	C. xerosis	
19	C. xerosis	
20	C. xerosis	
21	Lactobacillus spp.	
22	B. azotoformans	
23	C. xerosis	
24	C. xerosis	
25	B. insolitus/marinus	
26	C. xerosis	
27		
28	C. xerosis	
29	B. insolitus/marinus	
30	C. xerosis	
31	C. xerosis	
32	C. xerosis	
33		
34	C. xerosis	
35	M. varians	
36		
37		
38		
39		

40		

Figure 6: Table contains the identities of the forty bacterial samples determined from biochemical testing and DNA sequencing

Discussion

The biochemical tests yielded 20 different types of bacteria with 10 identified and 10 remaining to be identified. The identified bacteria are as follows *Corynebacterium xerosis, Enterococcus facecium, Micrococcus varians, Micrococcus luteus, Bacillus popilliae, Bacillus megaterium, Staphylococcus saprophytics, Bacillus azotoformans, Staphylococcus epidermidis, and Bacillus insolitus or Bacillus marinus.* The bacteria *S. epidermidis, S. saprophyticus, M. varians,* and *M. luteus* are normal inhabitants of the skin. Interestingly the bacterium *C. xerosis*, which is an inhabitant of the skin, has been known to cause animal diseases (4). The bacteria *B. megaterium, B. azotoformans, B. insolitus,* and *B. popilliae* are found in soil and thus their presence is unsurprising (1, 2). Lastly, *E. faceium* is a human pathogen which can cause surgical wound infections, endocarditic, and urinary tract infections (3). It is found in the gastrointestinal tract of mammals and can survive for long periods of time in the soil (3). It is unlikely that *E. faceium* or any of the bacteria listed so far are the cause of the bacterial infection in the horse population. Most likely these bacteria are part of the normal flora of the hoof and under normal circumstances they do not cause any issue, although they may take advantage of a weakened and/or infected hoof and begin to over reproduce. Hence their presence in the hoof samples.

The DNA sequencing determined the identity of seven bacteria. The identified bacteria are *Enterococcus gallinarum, Bacillus subtilis, Corynebacterium xerosis,* and *Macrococcus.* The bacterium *E. gallinarum* was the identity of three of the bacteria samples. As stated above, *C. xerosis* is an inhabitant of the skin and has been known to cause animal diseases (4). The presence of *B. subtilis* is understandable since it is found in the soil. The isolation of *E. gallinarum* and *Macrococcus* was very interesting. The bacterium *E. gallinarum is* very rare and had been located in the intestinal tract of mammals, although little else is known. *Macrococcus* is a relatively new division of classification in microbiology. Through research we discovered that one species of *Macrococcus, Macrococcus*

epuipercicus, has been found on horse's skin (5). Once again it is unlikely that the above bacteria are the cause of the hoof infection in the horse population.

As one may have noticed there are differences in the identities of the bacteria determined from biochemical tests (Crude ID) and DNA sequencing. The differences are as follows; *E. faecium* was mistaken for *E. gallinarum*, *C. xerosis* was mistaken for *E. gallinarum, M. luteus* was mistaken for *B. subtilis,* and *S. saprophyticus* was mistaken for *Macrococcus.* The misidentification of these bacteria could have been caused by misidentifying the morphology of the bacterial samples, unclear results of the biochemical tests, and contamination. We concluded from our research that using biochemical tests to determine the identity of a bacterium was a good method to get a general idea of the bacterium species, although DNA sequencing is a more accurate method. Furthermore, DNA sequencing allows one to identify bacteria that cannot be cultured. Therefore the infection causing bacteria may have not been isolated since we cultured all of the bacteria before sequencing the DNA.

Lastly, it should be mentioned that not all of the bacteria identified came from the infection site of the hoof. As stated in the method and materials, a sample was taken from the untrimmed area of the hoof to establish the general flora of the hoof. The bacteria isolated consisted of *C. xerosis, B. popilliae, B. megaterium,* and *B. azotoformans*. Only the bacterium *C. xerosis* was also isolated from the infection area of the hoof. Thus *B. popilliae, B. megaterium,* and *B. azotoformans* cannot be responsible for the infection in the hooves.

Conclusion

The exact cause of the hoof infection in the horses from Rochester NY has yet to be determined, although it is hypothesized that the infection causing bacteria are anaerobic and prefer moist environments. We hypothesize that the bacteria is anaerobic because the hoof infection improves when the hoof is trimmed in such a way that opens the infection area to the air. Also the hoof infection tends to clear up in the summer, when there is less rain. The bacteria causing the hoof infection weakens the white line of the hoof which allows secondary bacteria to flourish. The secondary bacteria probably obstructed the collection of a pure sample of the infection causing bacteria. Successful treatment and identification of the bacteria causing the hoof infection is essential because the hoof infection weakens the hoof allowing cracks to form in the wall and abscesses to develop. As of now, the best treatment method is the trimming of the hoof to expose the infected area to the air and treating weakly with iodine to control of the growth of bacteria.

Future Research

In the future, we hope to identify all the sampled bacteria using biochemical tests and DNA sequencing. From this we hope to determine the infecting causing bacteria, although it is likely further samples will have to be taken from the infected hooves. The collection method used to gather these samples will differ from the original method. Instead of using sterile, swabs we will use sterile needles which have a smaller surface area, to get a purer sample. Additionally, we will sequence the DNA of the bacteria without culturing them first in order to include bacteria that cannot be cultured. Once the infection causing bacteria have been identified then we will determine what conditions inhibit its growth, such as iodine concentration, to better treat the hoof infection.

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